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(54) Title: NONPATHOGENIC VARIANT VIRUS			
(57) Abstract Disclosure is made of nonpathogenic variant viruses, which may be generated through genetic engineering or which may arise spontaneously in a population of infected individuals. As specifically exemplified, the invention relates to nonpathogenic HIV variants which are competitive with pathogenic strains, and thus, nonpathogenic variant can be used to ameliorate the clinical condition of AIDS patients.			
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NONPATHOGENIC VARIANT VIRUS

5 This application is a Continuation-in-Part of U.S. Patent Application Serial No. 07/540,529, filed June 19, 1990, and U.S. Patent Application Serial No. 07/625,958, filed December 11, 1990, both of which are incorporated by reference herein.

TECHNICAL FIELD OF THE INVENTION

10 The field of this invention is the area of nonpathogenic variant viruses. More particular, the invention relates to spontaneously arising mutant viruses, specifically exemplified by those variants of Human Immunodeficiency Virus which are not pathogenic and which can be administered to
15 Acquired Immune Deficiency Syndrome patients to generate an immune response and to ameliorate clinical condition as well as the course of the disease.

BACKGROUND OF THE INVENTION

20 A significant number of infectious diseases in humans and animals are caused by viruses. A virus is composed of genetic material, either DNA or RNA, surrounded by a protein coat. Replication of a virus requires the help of a living cell. At least the genetic material of the virus, and in some cases other components, enter a living cell which then
25 accomplishes replication of the virus genetic material, synthesis of various viral proteins, including the proteins infected cell which, depending on the infecting virus, may be

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killed by the infection. The released viruses exist in an essentially vegetative state until such time as a virus encounters a susceptible cell, reinitiating the cycle.

5 A large variety of viruses infective to humans or animals is known. Both pathogenic and nonpathogenic viruses exist, although those most studied are the pathogenic viruses. Among the pathogenic viruses, strains of varying degrees of virulence are commonly observed. The mechanisms by which viruses exert their pathogenic effects are not well understood.

10 For one group of viruses, the retroviruses, their manner of replication appears to be responsible for their pathogenicity. The genetic material of retroviruses is RNA which, during infection becomes reverse transcribed into DNA, which integrates in the host cell chromosome. The virus becomes, in

15 effect, a component of the host cell. Pathogenic effects occur if the virus carries a gene which is harmful to the host. For non-retroviruses, the pathogenicity appears to be a consequence of the cytotoxic effects of a virus infection on the particular class of cells which the virus is able to infect. For example,

20 the paralysis which sometimes accompanies poliovirus infection, appears due to the ability of the virus to infect and kill peripheral nerve cells. In the case of human immunodeficiency virus (HIV), the virus is particularly cytotoxic to T-cells. It is the destruction of the T-cells in HIV-infected patients

25 which leads to breakdown of the immune function in the condition known as AIDS.

Strategies for dealing with virus disease have focused most intensely on immunization. In the classic work of Edward Jenner, immunity to smallpox was conferred on the subjects

30 exposed to cowpox, a related virus which, although pathogenic in cattle, functions as an attenuated strain in humans. Other examples of vaccines based on attenuated strains are known, most notably the Sabin vaccine for polio. In the absence of the fortuitous discovery of a naturally occurring attenuated

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strain, attempts to generate an attenuated live virus have been based on repeated serial passage of the virus through susceptible cells or tissues. That technique can only be employed if susceptible cells or tissues are available. The approach has also been limited by the suspicion among many researchers that mutation rates in viruses are so high that, in the absence of a selection procedure, the likelihood of finding an attenuated strain is extremely small. One rationale for the production of attenuated strains is that serial passage somehow permitted a series of mutations to accumulate, each contributing in a minute way to loss of pathogenicity without compromising viability. Since such mutations would not be expected to accumulate in the absence of some selective force which favors viability in the culture system, the latter is, in effect, a form of selection pressure imposed by the investigator. For example, in the case of polio, the serial passage through thousands of chick embryos could have resulted in selecting a poliovirus strain that was better adapted to replication in chick embryo through one or more mutations while simultaneously becoming nonpathogenic to humans.

Recently, however, Ubertyni et al. (1988) Vaccine 6:481, have shown that the Sabin Type III strain of poliovirus, widely used as an oral vaccine, differs from the neurovirulent "Leon" strain only at nucleotide position 472. The Leon strain has a cytidine (C) where the Sabin Type III strain has uridine (U). The Sabin Type III strain is known to revert to pathogenicity after passage through a vaccinated individual, and has been implicated in the vast majority of reported paralytic cases of poliomyelitis in the U.S. and U.K. (Evans et al. (1985) Nature 314, 548; WHO Consultive Group on Poliomyelitis Vaccines, Geneva, 8-10, July, 1989). The single base change which results in loss or gain of virulence is in a non-translated region of the viral genome. Ubertyni et al. (1988) also reported the discovery of a stabilized variant of Sabin Type III which failed to revert to pathogenicity,

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apparently by sporting a second, compensating mutation such that a single-step reversion at nucleotide position 472 fails to restore neurotoxicity. No mechanism or rationale to explain these phenomena has been published. However, the seemingly random and unpredictable nature of these events suggests that systematic methodology for obtaining and stabilizing attenuated virus strains is currently unavailable. It is well established since the definitive work of Luria S. E. and Delbrück, M. (1943) Genetics 28:491 that mutation precedes selection. The attenuated virus is not created by the passage conditions, it is merely selected from a pre-existing pool of variants that have occurred by spontaneous or induced mutation. Heretofore, a fortuitous choice of passage conditions was the key to successfully isolating an attenuated virus.

The human immunodeficiency virus (HIV) is the causative agent of a fatal disease, the acquired immune deficiency syndrome (AIDS). The epidemiology of the disease suggests that HIV is usually transmitted as a blood-borne disease and also by intimate sexual contact. Although certain groups in the U.S. are currently more severely affected by the disease than others, the entire population is at risk. No cure is known and no effective vaccine is currently available.

SUMMARY OF THE INVENTION

The present invention serves to improve the current situation by providing a systematic methodology for isolating nonpathogenic variant virus strains and stabilizing them against reversion so as to provide safe, reliable vaccines. A systematic theory is presented which predicts that attenuation generally results from a single base change in the absence of experimentally increased selection pressure. Consequently, attenuated strains can be found in any population of infected individuals, provided the population is large enough that the mutation will have occurred, given natural

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5 mutation frequency. Once a nonpathogenic variant strain is identified and its altered nucleotide sequence characterized, reversion to pathogenicity can be essentially prevented by introducing a second site, stabilizing mutation. The existence
10 of nonpathogenic variant HIV strains at a frequency of about 1 in 500 was predicted by the theory described herein, and at least two have now been found in a well-characterized HIV patient population. Following the teachings herein, the nonpathogenic variant HIV strains disclosed herein can be genetically modified to yield a live vaccine against HIV
15 infection. The nonpathogenic variant HIV strains described herein successfully compete against the pathogenic strain. A patient can be treated by superinfection with a nonpathogenic variant strain.

15 BRIEF DESCRIPTION OF THE FIGURE

Figure 1 displays a matrix which shows the decoding of the N-terminal sequence of gene V of bacteriophage f1, and the mirror-image patterns within the coding sequences. There are only two possible palindromic codings for such a sequence, one
20 of which contains internal-terminators.

DETAILED DESCRIPTION OF THE INVENTION

The following terms are used herein as defined: An attenuated virus is a strain of virus that is capable of infecting host cells and inciting an immune response in a non-immune infected host, but produces no symptoms or only mild
25 symptoms in an infected host. For example, Sabin Type III poliovirus normally produces an asymptomatic infection in a non-immune human. Cowpox virus normally produces only a single mild lesion at the site of vaccination. Both are examples of attenuated virus strains. Both induce an immune response in
30 vaccinated individuals, which confers protective immunity against pathogenic strains.

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5 The term pathogen is used to characterize an organism
that causes disease in a non-immune animal or human. A virus
is pathogenic if it causes disease. There can be differing
degrees of virulence associated with different strains of a
virus, depending on the severity of the clinical symptoms that
10 accompany infection of a non-immune animal or human by the
various strains. There will be a range of symptoms and
variations in severity among infected individuals, animal or
human. Assessment of pathogenicity and/or virulence is
15 therefore a matter of clinical observation, skill and
experience. While not strictly quantitative, an ordinarily
skilled clinician can evaluate the pathogenicity and virulence
of a virus strain using categories such as asymptomatic, mild,
moderate, moderately severe, severe, and the like. Therefore,
one ordinarily skilled in the art can identify and distinguish
infections by a nonpathogenic virus strain from those by a
pathogenic strain. For the purposes of this application, the
terms pathogenicity and virulence are used interchangeably.

20 A nonpathogenic variant strain is a mutant of a
pathogenic strain which differs from the latter in that it is
nonpathogenic, or is markedly less pathogenic, than the
pathogenic strain. A nonpathogenic variant strain may be
competitive with a pathogenic strain in an infected patient;
if so, it can become the predominant strain isolated from an
25 infected patient. This will occur especially in the case of
viruses which are sufficiently able to evade the immune system
so as to establish chronic infections. A patient in whom a
competitive nonpathogenic variant has arisen or has been
introduced will eventually fail to yield detectable pathogenic
30 virus, since the nonpathogenic variant will have outgrown the
pathogenic strain.

Immunity is the state achieved in an individual animal
or human such that infection by a pathogenic virus results in

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an asymptomatic infection or an infection of reduced severity. Immunity is usually the result of stimulating production of antibodies, usually against coat protein or other external components of the virus. In order to provide protective immunity, a protein of an attenuated virus must elicit antibodies that bind and inactivate pathogenic strains. The antibodies raised against the attenuated strain must react with the pathogenic strains essentially as well as with the attenuated strain. The antibody reactivity can be readily compared by known in vitro tests, which will be predictive of in vivo effectiveness. In the case of a genetic change outside the structural gene of the virus, the immunological reactions of the attenuated strain will be identical to pathogenic strains, both in vitro and in vivo.

Genotypic selection is the term used to describe the process of selection at the nucleotide level for efficiency and stability, by operation of the biochemical processes of replication, transcription, reverse transcription, translation and expression. Selection at the genotypic level can lead to convergent solutions such that nonpathogenic mutants can be selected for in the absence of an artificial phenotypic selection. Genotypic selection is the means whereby nonpathogenic mutants arise in the absence of an artificial selective environment. The concept of genotypic selection has been described by Pieczenik, G. (1980) J. Mol. Biol. 138:879-884; Pieczenik, G. (1980) Proc. Nat. Acad. Sci. 77:3539-3543; and Crick, F.H.C. et al. (1976) in Origin of Life, Vol. 7, 389-397. Briefly, it is proposed that there are features of nucleotide sequences which confer greater stability, or greater efficiency of expression, than others, given that there are many possible sequences for expressing and controlling expression of the same set of proteins. Just as in computer software or plain English, there are many alternative ways to convey the same information, but some are more efficient, or economical, than others. The basic principle of genotypic

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selection is that those nucleotide changes which lead to more efficient expression, or to greater stability of the genome, will have an adaptive advantage independent of the environment. Therefore, random mutations that lead to such adaptive advantage will be selected for and will propagate through the population of organisms. Similarly, synonymous nucleotide changes do not fix randomly in the population without selection.

Evolutionary convergence occurs at the genotypic level, according to the theoretical discussions of Crick et al. (1976) *Origins of Life* 7:389-397; Pieczenik (1980) *Proc. Natl. Acad. Sci. USA* 77:3539-3543; Pieczenik (1980) *J. Molec. Biol.* 138:879-884. Selection acts directly on nucleic acid sequence and structure via the biochemical machinery for replication, transcription and translation. Genotypic selection can result in constraints which are observed as nucleotide sequence patterns of various kinds. Patterns can be generated from a series of constraints. If selection is sufficiently strong and if several selective mechanisms act on the same nucleotide sequence, then convergent and predictable solutions are possible, given that nucleotide sequence changes are independent of one another, and only changes that are expressed to give the whole organism or virus a reproductive advantage are preserved.

An example of this type of prediction was first presented in Pieczenik et al. (1972) *J. Mol. Biol.* 90:191-214, which suggested that there were at least two types of nucleotide constraints, both a consequence of selection at the nucleotide level. One was called the internal-terminator constraint, which held that there was a selective advantage in having a terminator out-of-phase in the beginning of a sequence (Pieczenik et al. (1972) *Biochem. Biophys.* 152:152-165). The use and suppression of internal-terminators for read-through regulation of protein translation is now a common observation

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in many viral and bacterial systems, and is presently seen in HIV sequences.

5 An interpretation of the second constraint, a true palindromic single-stranded constraint, held that "single-strandedness" would confer an advantage to loop regions of loop-stem structures and to the sides of double stranded structures. Random RNA sequences can be folded into extensive secondary structures given AU, GC, and GU base-pairs. Genotypic selection for "single strandedness" would give these
10 randomly folded structures adaptive shapes.

The first DNA sequences contained true palindromes, i.e., Mutate E. Tatum or the phi X 174_G sequence ATG.TTT.CAG.ACT.TT; phi X 174_F T.GCT.GGT.CAG.ATT.GGT.CGT.

15 The independent existence of the internal-terminator constraint (a syntactical constraint) and the palindromic single-strand constraint (a physical constraint) implies independent selection for these sequence constraints. However, if genotypic selection for internal-terminators and palindromicity act on the same nucleotide sequence, the
20 evolutionary options for such a sequence become extremely limited.

For example, for all sequences that are eighteen nucleotides long and contain internal-terminators palindromically arranged, there are only 27 possible sequences,
25 i.e., URRURRURR/RRURRURRU, where R is A or G, but RR cannot be GG. There are 3 terminator sequences. Therefore, there are $3 \times 3 \times 3 = 27$ sequences that are each 9 nucleotides long. Since it is a mirror image sequence, the other 9 nucleotides are fixed and therefore there are only 27 such possible
30 sequences.

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On the other hand, the total number of sequences that are 18 nucleotides long equals $4^{18} = 68,719,476,736$. Imposing two such weak constraints on the same region of nucleotide sequence allows a reduction in evolutionary options for that sequence of $27 \div (4^{18}) = 3.93 \times 10^{-10}$. Therefore, proposing concerted genotypic selection allows for a ten billion-fold potential reduction in evolutionary options for a nucleotide sequence, independent of protein constraints.

One of these 27 predicted sequences codes for the ribosome binding-site of f1 bacteriophage's single-stranded DNA binding protein gene V, i.e., fMET-ILE-LYS-VAL-GLU-ILE-LYS coded by AUG-AUU-AAA-GU/U-GAA-AUU-AA. This is the first time a nucleotide sequence was predicted to exist a priori and such predictions are strong evidence for direct genotypic selection. This argues against the Kimura-Jukes theories which would require that all synonymous codings for a particular protein have the possibility of existing equally frequently.

The average total number of synonymous codings for a protein is $61/20X$, where X is the amino acid length. Thus in a 5 amino acid sequence, one should find 263.9 (3.05^5) or, by directly multiplying synonymous coding options ($3 \times 3 \times 4 \times 2 \times 3 \times 2$), 288 possible codings. Fig. 1 is a matrix that shows the decoding for f1 bacteriophage gene V protein's amino terminus and the mirror-image patterns contained within these codings. It shows only two palindromic codings for such a sequence, one of which contains internal-terminators.

This suggests that neutral amino acid substitutions and particularly that synonymous codon replacements are evolutionarily selectable. Therefore, an examination of synonymous codings for identical proteins in different organisms, or even nucleotide polymorphisms, can decide whether there is selection at the nucleotide level which will lead to convergent coding solutions.

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Another example of Genotypic Selection is the selection imposed by the existence of GU base-pairs through mutation. Unlike AU and GC base-pairs, GU base-pairs were originally postulated by Crick as necessary for his wobble hypothesis. Later, Crick et al., supra, showed that the base-pairing interactions between tRNA and mRNA evolved from a 5 base-pair interaction that employs the conserved U, which is 5' to the anti-codon in all tRNA molecules (except fMet tRNA) in potential GU base-pairs. This model allows for a flip of the anti-codon loop which reads the mRNA three bases at a time (5' to 3') though there are five bases involved in each tRNA-mRNA interaction. A five base-pair RNA interaction is relatively stable. The sequence constraint that this type of model of translation imposes on the evolutionary history of mRNA, as a consequence of GU base-pairing, is a R,N,Y (purine, any nucleotide, pyrimidine) bias on coding sequences.

RNA interactions of the type described above, i.e., intramolecular base-pairing in loop-stem structures and inter-molecular base-pairing in tRNA-mRNA type interactions, or snRNA splicing interactions, rely on the structural fact that GU base-pairs occur and are acceptable base-pairs in these interactions. The structural stability of a GU base-pair in a hairpin structure is significantly lower than that of a GC or an AU base-pair. The energy and shape of a GU base-pair is such that it cannot stabilize short stretches of base-pairing. As five base-pairs are independently stable, a GU base-pair flanked by two 5 base-pair stretches would be stable. However, a GU base-pair flanked by two 3 base-pair regions would not be stable. Therefore, a 7 base-pair region that mutates its middle base-pair to a GU base-pair would go from stable to unstable, whereas an 11 base-pair region having mutated its middle base-pair to GU would still be stable.

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5 This argument puts a minimum size of 11 bases on a side
of a loop-stem structure for a mutation of a middle base-pair
to a GU base-pair not to detectably change the stability of the
structure. Because this is a loop-stem structure, i.e., 22
nucleotide bases as the minimum interaction target, it is not
detectably affected by a GC or AU to GU base-pair mutation.
This target size could possibly go down to 18 (nine on each
side) and still be stable, but 14 nucleotides (seven on each
side, i.e., 2 three nucleotide base-pairs surrounding a GU
base-pair) is not at all stable.
10

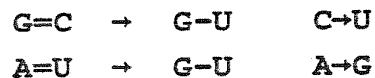
Therefore two 4 to 5 GC or AU base-pairs surrounding
a GC base-pair or an AU base-pair that mutates to a GU base-
pair is a stable, relatively non-detectable structural change
in RNA-RNA interactions. This means a minimum target size of
15 18 to 22 nucleotides (or 20 nucleotides) on average.

In summary, any RNA interactions that are necessary to
preserve contiguous structure must have a minimum of 20 base-
pairs so as not to be disrupted by the mutation of a GC or an
AU base-pair to a GU base-pair. While the stability and shape
of GU base-pairs in RNA structures can give an estimate of the
20 minimum size required for structural stability of RNA
interactions involved in mutating to GU base-pairs from non-
GU base-pairs, the relative frequency of GC and AU base-pairs
in these structures and interactions can give us mutation
25 direction.

GC and AU base-pairs occur in RNA interactions and RNA
structures at a higher frequency than GU base-pairs.
Therefore, there is an asymmetry in mutation direction of AU
base-pairs and GC base-pairs to GU base-pairs. This appears
30 at a higher frequency of C to U, as opposed to U to C
transitions and also, a higher A to G transition frequency than
G to A. This gives a direction to the evolution of RNA

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structures and interactions; that is, C to U and A to G, as follows:



5 This model of evolutionary direction and stability of
structures suggests that bacteriophage f2 is convergently
evolving into MS2 which is convergently evolving into R17. In
addition, Fiers noted that during the course of sequencing MS2
coat protein, a CAA(GLN¹⁰⁹) codon was seen to mutate (and fix
10 in the population of sequenced phage and molecules) to a CAG
synonymous codon -- again an A to G transition (Fiers, W.
"Chemical Structure and Biological Activity of Bacteriophage
MS2 RNA", in Zinder (ed.) (1975) RNA Phages, Cold Spring Harbor
Press, Cold Spring Harbor, N.Y., p. 386. This can be explained
15 as an AU base-pair mutating to a GU base-pair.

 This is the first time one can put a logic and
direction to synonymous mutations, showing evidence of
convergence at the nucleotide level. Since the changes
observed were synonymous and did not affect protein sequence,
20 this cannot be a consequence of protein function. It
demonstrates competition and fixation between nucleotide
sequences and sequence structures, independent of sequence
expression.

 The present invention is based on four fundamental
25 insights: 1) that a loss of pathogenicity is not necessarily
maladaptive for a virus, 2) that a single base change can
confer loss of pathogenicity, 3) that such a base change can
occur by genotypic selection, and 4) that such a change, once
identified, can be stabilized by an introduced second site
30 mutation that effectively presents reversion to pathogenicity.
As a consequence of these insights, it is now possible to state
that with a statistical frequency of about 1 in 500 virus-
infected individuals, a strain of nonpathogenic virus arises,

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and by screening a population of virus-infected individuals, nonpathogenic strains are found which serve as nonpathogenic variant virus strains for vaccine production. A corollary of this finding is that the nonpathogenic variant virus isolatable from an infected individual is competitive with pathogenic virus, since the variant strain is able to multiply in sufficient numbers to be detectable in the individual, and since the individual is initially identified by lacking symptoms of pathogenic infection. As described in detail herein, a population of about 1500 HIV-positive patients has been followed clinically for over 10 years. A few individuals were identified who had been HIV-positive for over 10 years but had remained clinically asymptomatic, with normal T-cell counts. HIV could be grown from the blood samples of some of these patients. However, in these cases, the HIV strains grew more slowly than normal and did not appear to produce the cytopathic effects in culture characteristic of pathogenic HIV. We have identified at least two nonpathogenic variant HIV strains. Following the teachings herein, these strains can be further modified by specific second site mutations, genetically engineered to stabilize the attenuated strains against reversion to pathogenicity. In addition, the course of infection can be altered by superinfecting patients with a competitive nonpathogenic variant strain.

In order to teach how and where to make the second site mutation, it is necessary to explain how a single nucleotide change arising by genotypic selection can result in loss of pathogenicity. The genomes of many viruses are single-stranded RNA or DNA. All viruses make one or more m-RNA's which are single-stranded. Genotypic selection can act to optimize stability of these single-stranded DNA's or RNA's.

It is well-known that single-stranded nucleic acids can form a secondary structure, using base pairing interactions between nearby sequences. For example, two segments that

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happen to be complementary with each other can form a "stem-loop" structure, the two segments forming a locally base-paired stem, while the segment between them forms the loop. Such structures are frequently observed; their stability increases as the number of base pairs forming the stem is increased. Two RNA molecules can interact with one another in a similar manner through the interaction of segments of locally complementary sequence. Since single stranded nucleic acids are flexible molecules under physiological conditions, stem-loop structures can tolerate some unpaired bases on either side of the stem, since these can simply exist puckered out of the stem structure.

At least five base pairs are required to stabilize a stem-loop structure. Longer stems, having more base pairs, have greater stability. A mutation in the stem which interrupts base pairing can destabilize a short stem, but can be tolerated by a longer stem. Mutations that destabilize existing stem-loop structures are less adaptive. One type of mutation that can be tolerated in a stem-loop structure is the type that results in forming a G:U pair, either by an A to G mutation in an A:U pair or by a C to U change in a G:C pair. G:U pairs can form a single hydrogen bond which contributes somewhat to stem-loop stability, provided the G:U pair is flanked by 4 or 5 A:U or G:C pairs. A stem-loop structure of about 20 nucleotides total length will therefore be long enough to tolerate the appearance of a G:U pair by mutation without being selected against by genotypic selection (Nussinov et al. (1984) J. Theor. Biol. 106:245-259; Ibid. pp. 261-273).

The mutation may nevertheless have a phenotypic consequence, as by affecting pathogenicity. A mutation of this sort can persist in a population and the resultant strain can be nonpathogenic. It can also revert, by a reversal of the original mutation to restore the pathogenic sequence. Once the change is identified, however, one skilled in the art can

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introduce a corresponding base change in the previously unchanged member of the pair, to maintain the stem-loop integrity but virtually eliminate the probability of reversion to pathogenicity. For example, if the initial (pathogenic) sequence was G:C and a C to U mutation resulted in a nonpathogenic strain, the resulting G:U pair could be further modified by a second site mutation of the G to an A, giving an A:U pair. The second mutation maintains stem-loop stability together with the nonpathogenic phenotype. Reversion of the U to C would restore pathogenicity but would destabilize the stem-loop, resulting in genotypic selection against such a revertant, and failure to propagate in the population of viruses. Other corresponding mutations, such as deletions, can also prevent viable reversions.

The foregoing discussion has been couched in terms of the example of a stem-loop structure. Other more complex structures are known to exist as the result of localized base-pairing interactions in single-stranded nucleic acids. These also function as potential loci for genotypic selection. The majority of the base pairs in such regions of secondary structure are A:U and G:C pairs. Because mutations to G:U can be tolerated within such structures, a bias will be observed in favor of A to G and C to U mutations in replicating RNA virus populations. Where several cycles of replication have occurred, such that a nonpathogenic variant has appeared, genotypic selection allows that variant to persist and even overgrow the original pathogenic strain.

Pathogenicity itself is generally considered maladaptive for a parasite. By killing a host, or essential cells within a host, a parasite limits the extent to which it can replicate within the host. Therefore mutations that prolong host viability, i.e., reduce pathogenicity, tend to be favored in evolution. The combination of genotypic and

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phenotypic selection pressures leads to the existence of nonpathogenic variant strains in replicating virus populations.

Genotypic selection operates without regard to phenotype. It will therefore be understood that genotypically selected variants will arise that have no apparent effect on phenotype. Also, the phenotype may be affected by a mutation but the new phenotype may not confer any increase in fitness, except for that conferred by genotypic selection itself. Therefore, the improvement in fitness of a competitive nonpathogenic variant does not depend on the phenotype of nonpathogenicity, although that phenotype may further contribute to fitness.

The frequency with which nonpathogenic strains can be found in infected individuals can be estimated from the minimum target size of about 20 nucleotides, as discussed supra, which can accept a G:U pair without disrupting secondary structure: one simply divides 20 by the total length of the viral genome in nucleotides. For poliovirus, the calculated frequency is $20/7431 = .0027$, the fraction of an infected population carrying a nonpathogenic strain of polio during an epidemic of the disease. The calculation is based on the assumptions that all sites on the virus are equally likely to mutate and that only mutations away from the original sequence in the 20 nucleotide target region associated with pathogenicity will disrupt whatever interaction has been lethal to the host cell. As has been shown with poliovirus, a C to U change can disrupt such a pathogenic interaction. Without further identifying or offering any hypothesis as to what the nature of the interaction is, it follows that any nucleotide change in the target region would be effective to inhibit the interaction and remove the pathogenic phenotype.

It is also understood that nonpathogenic variants may result from other mutational events, including other base

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substitutions, insertions and deletions. Deletion mutations offer the advantage that reversion is extremely rare.

The frequency of nonpathogenic variants of HIV can be calculated based on the same assumptions. The target size of 20 nucleotides divided by the genomic size of 8213 for HIV yields .0022. Approximately one patient out of 500 carries a nonpathogenic form of the virus according to the calculation. This analysis has been borne out by clinical data.

Analysis of clinical data for HIV-infected patients has been complicated by the long and variable latent period between infection, (an event which cannot always be accurately dated) and onset of symptoms, which are initially equivocal. The best indicator of infection is the presence of virus, detected immunologically, or of antibodies to the virus in a patient's blood. Indicators of pathogenic effects, other than full-blown AIDS, include significantly lowered T-cell count, lowered percentage of T4 lymphocytes, and clinical picture of lymphadenopathy and other symptoms associated with onset of immune deficiency. Systematic studies are further confounded by issues of confidentiality, compliance and longitudinal tracking in the major at-risk population sub-groups. The present study includes a closely-followed group of about 1500 patients, many of whom have been monitored for more than 10 years. A few individuals from this group were identified as having tested HIV-positive for at least 8 years and who were clinically asymptomatic, i.e., had normal T-cell counts and otherwise normal blood chemistry. Nevertheless, the presence of HIV in the blood of these patients could be demonstrated by "Western" blot gels showing presence of a characteristic HIV-coded protein, and by polymerase chain reaction (PCR) to amplify and identify the presence of HIV DNA.

The criteria for identifying individuals carrying a nonpathogenic HIV variant include: 1) positive reaction for

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HIV using Western blot or PCR assay, or both, 2) lack of clinical AIDS symptoms with relatively normal T4/T8 ratios and normal number and percent of T4 cells, 3) production of HIV virus in vitro, and 4) good health for a long time after infection. A further criterion can be the identification of those individuals meeting the above criteria plus the evaluation of the partners of those individuals who also have stable or stabilized T4 cell counts. The last criterion is particularly applicable when an anal receptive partner of a long-term healthy, HIV-positive individual with clinical symptoms of AIDS shows stabilization of T4 cell counts and percentages and stabilization of his condition (See Example 3 for further discussion).

The growth in culture of HIV from three of the anomalously asymptomatic patients was tested. The characteristics to be expected of an attenuated HIV strain are: 1) non-lethal to T-cells in culture; 2) independent of cells from a particular host (can grow on cells other than the donor's); 3) growth rate in culture slower than that of the pathogenic strain, possibly not observable without intervention of an inducer; 4) at least a one base (or base pair, if applicable) alteration in RNA sequence compared to wild-type pathogen. Two of the three patients' virus isolates were found to grow in vitro, however growth was slower than wild-type HIV. In the third case, no growth in culture was observed, although the presence of HIV in the patient's blood was confirmed.

Identifying the base changes responsible for nonpathogenicity is accomplished by sequence analysis of the RNA of each isolate. HIV is known to be somewhat variable in sequence, even as between pathogenic isolates. On the average, the number of sequence variants which can be isolated from a single patient is about nine (Ratner et al. (DATE?) Nature 313:277; Fischer et al. Science (1986) 233:655). The base change responsible for loss of pathogenicity can be identified

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by several strategies. For example, the number of possible candidates can be drastically reduced by eliminating those not located in stem-loop or other secondary structures and those within coding regions. By sequencing several of the nonpathogenic strains isolated from one patient and comparing those nucleotide sequences, the region where mutations to nonpathogenicity occur can be identified unequivocally. Hemophiliac populations can be tested, since these patients are a well-studied, high risk group for HIV infection. Given the number of known infected individuals, there are an estimated 2000 asymptomatic carriers of nonpathogenic HIV in the U.S. alone. For other viruses, the search for asymptomatic carriers is preferably concentrated in areas and at times of an epidemic. The frequency of occurrence of such individuals is about 20 divided by the genome size of the virus in question, as described supra, per infected individual. Since asymptomatic individuals are not readily identified as infected, the search is preferably conducted among those most at risk, i.e., members of a patient's household, school or place of work. For screening for avirulent HIV, the populations of sexual contacts and/or populations sharing needles for injected drug use can also be used. The choice of at-risk group is based on knowledge in the art of the mode of transmission of the virus and well-understood epidemiological principles. The frequency of finding asymptomatic carriers is less than 1/500, since any screening procedure is likely to include uninfected individuals. The actual frequency will depend in part on the state of knowledge in the art with respect to defining an at-risk population for each virus for which an attenuated strain is desired.

After identifying the locus of a base change yielding nonpathogenicity, one skilled in the art can introduce an appropriate second site mutation by known techniques, for example by site-specific mutagenesis or by polymerase chain reaction (PCR). Smith (1985) Ann. Rev. Gen. 19:423, PCR

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Protocols, (M. A. Innis et al. eds.) (1990) Academic Press. The appropriate mutation is one which stabilizes the original mutation by eliminating the possibility of base-pairing as a result of reversion at the locus of the first mutation, as taught, supra. Although a second site mutation of the type described could arise naturally, it would only be observed with a frequency comparable to that which generated the original mutant. Such a variant would arise only about once in every 500 individuals bearing a nonpathogenic strain. Therefore, it would be unrealistic and unacceptable to await discovery of such a variant. Therefore the use of the techniques of the present invention to generate such strains (termed a Highly Adaptive Nonpathogenic (HANP) strain herein) are of great benefit to providing a live virus vaccine. It would appear that the non-reverting Sabin strain described by Ubertini et al. is an example of a HANP strain of poliovirus.

By transforming appropriate host cells in culture with nucleic acid of the HANP strain, HANP virus can be generated and replicated to any desired extent.

Use of a HANP strain to produce immunity can be carried out either with live or inactivated virus. Live virus can be administered by conventional means, and can provide protective immunity at an acceptable risk level. In many instances, the degree of immunity can be monitored, if desired, by measuring the recipient's antibody titre at intervals after the vaccine is administered. In certain instances, for example, with HIV, an additional safety margin can be provided by administering HANP virus as an inactivated or killed virus. The amounts of killed virus to be administered, sufficient to provide immunity, can be determined without undue experimentation by monitoring antibody titres obtained after a series of trials with test subjects. Larger amounts of killed virus are needed to confer immunity compared to live nonpathogenic virus because the killed virus is unable to replicate in the host. Any

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technique known in the art can be employed for killing or inactivating a virus without destroying antigenicity of the coat protein or other surface proteins. The combined safety factors for HANP virus (conservatively estimated at 1 double revertant per 10^{11} to 10^{12} virus) and killed virus (1 active virus per 10^9 to 10^{10} inactivated particles) amount to an estimated 1 in 10^{20} to 1 in 10^{22} pathogens per administered virus. An administered dose of approximately 1g of virus is only about 10^{11} - 10^{12} particles, so only about 1 in 10^8 - 10^{11} vaccine doses would contain a single pathogenic virus. The foregoing estimates are approximate only and do not take into account biological factors which might bias the observed values in either direction; however, they indicate that a high degree of safety is inherent in the use of inactivated HANP vaccine.

A particular advantage of a live HANP vaccine is provided by the fact that such a virus can replicate in host cells without destroying them, whereas the pathogenic strain destroys the host cells. Therefore, a HANP strain can replicate and ultimately stimulate a protective immune response even in a patient already infected with a pathogenic strain. This advantage can be observed only in the case of a viral disease that is slow to develop as compared with the time necessary to mount an immune response. In the case of HIV, the advantage can be observed in early and middle infection stages, but will not be observed if the patient is too severely immune-compromised to form antibodies. Further, in the case of HIV and other viruses which evade the host's immune response, either the nonpathogenic variant or an HANP strain can compete with and replace the pathogenic virus in a host infected with pathogenic virus. Consequently, a patient who is experiencing symptoms of the disease can be superinfected with a nonpathogenic variant strain and thereby be protected against further ravages of the disease as the nonpathogenic variant becomes the predominant, or only strain in the patient. It will be understood that the outcome of such superinfection will

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be influenced by the extent of permanent damage suffered by the patient at the time of superinfection.

5 The following examples serve to further document the techniques and data underlying the present invention. Sampling of blood, and analyses of blood samples and isolation of T cells were carried out by standard clinical methods. The presence of HIV in patient blood samples was verified by both immunoassay and by identifying HIV DNA by polymerase chain reaction (PCR). All methods of virus culture were standard methods, used in accordance with published protocols.

10 A group of about 1500 individuals testing HIV-positive has been tracked over a period of more than ten years. Although longitudinal studies of HIV-infected patients are sometimes difficult in practice to carry out, they are not impossible or unique. Certain groups of at-risk individuals, for example hemophiliacs, are relatively easy to study longitudinally; indeed several such groups are currently under study. Other at-risk groups, such as intravenous drug users and homosexuals, present a greater challenge to study over the long term due to higher mobility, and the difficulty of establishing mutual trust between patient and investigator. The present investigation involved a group of patients who were not preselected as to risk category, but rather were initially identified as HIV-positive and subsequently monitored for the appearance of clinical symptoms in order to commence early and appropriate treatment. While the thrust of the study was for therapeutic purposes, records of standard tests performed over the years were kept to develop a more comprehensive picture of the disease during the course of infection.

25 Three individuals identified as testing positive for HIV over a period of ten years or more without having clinical symptoms of immune deficiency were selected for the in vitro studies. These individuals displayed normal T-cell counts.

30

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While the T-cell count varies over a wide range of normal values, a reduction in T-cell count of more than 50% below an individual's baseline count (e.g., below 530) or a dramatic increase in the T8/T4 cell count ratio over the normal ratio is a strong indicator of onset of immune deficiency. From two of these individuals, nonpathogenic HIV variant viruses were cultured. One nonpathogenic HIV variant was used to inoculate a set of test patients, with mixed clinical results. In all test patients, however, there was seen increased production of antibodies specific for HIV components and increased cellular immunity functions. The best positive correlation of improved clinical outcome with a parameter measured in this study was for the production of antibody specific for HIV core protein p15.

Further study indicates that the donor's serum and the sera from the four test patients with improved health shows strong binding to all HIV proteins, and in particular to 15kDa (p15) in the Roche assay and 17kDa doublet (p17) in certain other Western blot systems. Generally sera from test patients who exhibit mixed response or a decline in health do not bind to the p15 or p17 doublet on Western blots. Severely immunocompromised HIV-positive individuals with AIDS no longer exhibit antibodies specific for p15 and p17 by the Western blot assay. Thus, the production of antibody(ies) specific to p15 (or to the p17 doublet) is positively correlated with the presence of the putative competitive, nonpathogenic HIV variant of the present invention, and is a predictor, albeit not absolute, of improved clinical condition. It is understood in the art that apparent molecular weights may vary, according to the exact conditions used for polyacrylamide gel electrophoresis.

Other clinically useful strains may have other phenotypes with respect to the antibodies generated. It will be understood by the skilled artisan that p15- (or p17

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doublet-) specific antibody production is not an absolute predictor of clinical utility. However, the disclosed nonpathogenic variant virus from the donor (corresponding to IMM-1) has had a 40% success rate in the test patient population as determined by improved health. Improved health in those test patients is correlated with increased response in delayed-type hypersensitivity skin tests, improved sense of well-being and return to a normal life style.

Epidemiological studies have identified fifty additional potential donors, including twenty from the 1978 San Francisco Hepatitis B Cohorts Study. From independent serological studies on potential donors, five potential donors whose sera bind to the p15 and/or p17 doublet on Western blots of HIV proteins were identified.

For reasons of confidentiality all patients described herein are identified by patient numbers.

Deposits have been made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, M.D. 20852. Those deposits were of fresh frozen peripheral blood mononuclear cells containing nonpathogenic HIV variants. The deposit identified as Immuvax 1 (IMM-1) corresponds to Patient #1, as described in Example 2. Deposits identified as IMM-29 and IMM-41 are from two other potential donor individuals (IMM-29 and IMM-41, respectively) who have tested as HIV-positive and have remained free of ARC or AIDS symptoms for at least eight years. The blood from IMM-1 (Patient #1) has yielded HIV variant virus which does not kill infected cells in cell culture, as described in Example 1. While in vitro attempts to culture nonpathogenic variant HIV from samples IMM-29 and IMM-41 have not yet been made, these blood cell samples are candidate sources of competitive nonpathogenic HIV variant strains.

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Example 1. HIV replication in vitro.

Blood samples of patients 1, 2 and 9 and control patients were obtained with consent. T-cell [HIV] preparations from each sample were used to inoculate in vitro cultures of T-cells from four pooled normal (HIV-negative) healthy donors. A total of 2×10^7 patient's cells were added to 2×10^7 pooled cells from buffy coats of four healthy donors (purchased from American Red Cross and stored at -80°C . until use). Pooled T-cell preparations were used to eliminate variations in growth possibly caused by an unknown bias in individual virus isolates for individual T-cell types. The same preparation of pooled cells was used as in all HIV growth studies. All reagents and media were obtained from commercial sources. Abbreviations used herein are standard in the art.

The culture medium contained 80%(v/v) RPMI 1640, 20%(v/v) 199 Earle's salts 10%(v/v) Fetal Calf Serum (FCS) inactivated by incubating 30 min at 56°C , 1%(w/v) human albumin, 100 IU/ml penicillin, $100\mu\text{g/ml}$ streptomycin, 0.05 mg/ml gentamicin. Pooled cells were suspended in RPMI 1640 at $10^7/\text{ml}$ and incubated with 1.25mM L-leucine methylester (Leu-O-Me) Sigma Chemical Co., St. Louis, MO, for 10 min at room temperature. Cells were then washed three times in RPMI 1640 plus 2%(v/v) FCS and then co-cultured with patient's cells.

Three types of culture were tested, varied according to the type of stimulated T cells employed. MLR ("mixed lymphocyte reaction") cultures contained pooled cells that were pre-stimulated with phytohaemagglutinin (PHA) for three days before they were co-cultured with patient's cells. Additionally the culture medium contained 50 U interleukin-2 (IL-2). sPHA-T cultures contained 25%(v/v) of a supernatant of irradiated and 24h PHA-stimulated human T cells. sPWM-T cultures contained 25%(v/v) of a supernatant of irradiated and 24h pokeweed mitogen (PWM)-stimulated human T cells. Every 5

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5 days, 1ml culture medium plus PBL was replaced with 5×10^6 pool cells in 1ml medium. For MLR cultures, pooled cells were prestimulated with PHA for 3 days. For the other cultures pool cells were Leu-O-Me treated and the 1ml contained 25% SPHA-T or SPWM-T, respectively.

Growth of HIV in culture was measured by the quantity of p24 antigen, using HIVAG-1 kits (Abbott Laboratories) for p24 detection and the HIV-1 p24 antigen quantitation panel (Abbott Laboratories) to generate a p24 standard curve.

10 Example 2.

15 Over many years of clinical experience of a patient group numbering more than 1500 individuals, a small number of patients presenting an anomalous clinical picture were observed. These patients had been infected with HIV for more than eight years but remained essentially asymptomatic and had maintained normal T-cell counts. From an initial screen of apparently anomalous cases, the following were selected for further analysis:

20 Patient #1-, a 34 year old male; date of infection, 1979. The patient has never experienced any symptoms of immune deficiency. Current tests for HIV DNA by PCR were positive. Current Western blots tested positive (+++) for antibodies to gp120, gp41 and p24. Tests for p24 antigen were negative. In 1984, the patient's T4 lymphocyte count was 900: in August, 25 1989, the count was 1190 (35% of total T cells), and T8 lymphocyte count was 1496 (44%). Currently, the T4 count is 1085 (35%) and the T8 count is 1364 (44%). Other indicators, including sedimentation rate and lymphocyte counts, were normal.

30 Patient #2- a 41 year old male; date of infection, 1979. The patient has had no clinical symptoms attributable

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to immune deficiency except for a mild thrush episode in 1985. A PCR test for HIV DNA 6 months ago (11/89) was positive. Western blot analysis for antibodies to gp 120, gp41 and p24, conducted at approximately the same time, was positive, although higher levels were observed when the test was repeated thirteen months later. A test for p24 antigen was positive (75pg/ml) in September 1988, but negative thirteen months later. In 1987, the patient's T4 lymphocyte count was 576 (36%), but had increased to 902 (41%) by October, 1989. His corresponding T8 lymphocyte counts were 480 (30%) and 880 (40%), respectively. Other parameters were normal.

Patient #9- a 38 year old male; date of infection, 1981. This patient began having minor lymphadenopathy early in 1987 and had an episode of seborrheic dermatitis in November, 1987. He is currently asymptomatic. The patient currently displays a positive PCR test for HIV DNA, and has antibodies to gp120, gp41 and p24 (+++) as shown by Western blot analysis. The test for presence of p24 antigen was negative. The patient's 1986 T4 lymphocyte count was 304, which was elevated somewhat to 410 (25%) in early 1987, and further increased to 650 (26%) currently. His T8 lymphocyte counts were 960 (58%) in early 1987, and 1500 (60%) currently. Total lymphocyte count increased from 1650 to 2500 over the same time period. Other parameters were normal. This patient has received AZT treatment since 1988.

Blood samples from patients #1, #2 and #9 were cultured in vitro , as described in Example 1, to determine whether virus could be cultured from these patients and to test the growth characteristics. The results are shown in Table 1. Shown for comparison is the data of patient #11, who has a low (40-100) T4 count and lymphadenopathy symptoms.

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Table 1
p24 (pg/ml)

<u>Patient #</u>	<u>Day 5</u>	<u>Day 10</u>	<u>Day 15</u>	<u>Day 20</u>
1	2,743	56,897	23,688	22,864
2	-0-	-0-	-0-	-0-
9	233	5,277	12,953	13,553
11	20,500	124,213	51,424	100,600

In the case of patient #2, no virus protein was detectable in culture. Virus protein was detectable from culture of patients #1 and #9, after a lag period. For comparison, a blood sample from a patient infected with pathogenic virus (#11) yielded significant levels of virus protein as soon as 5 days after inoculation.

Example 3. Symptom control by superinfection with a nonpathogenic variant HIV.

Patient #15 is a homosexual male who has been asymptomatic for 10 years, despite a date of infection estimated at 1979 or early 1980. Patient #16 has been infected since 1978. Patients #15 and #16 reported that they have been lovers since 1978, with multiple contacts outside the relationship until 1984. Since 1984 to the present, their relationship with one another has been exclusive (monogamous). Their pre-1978 lovers are now dead. Both men have continuously tested positive for antibodies to HIV proteins by Western blot and negative for p24 antigen. Both men have continuously tested positive for HIV DNA by the PCR test. In their sexual activity, patient #15 has the masculine "inoculator" role, patient #16 is passive (anal receptive).

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In 1982 patient #16 developed symptoms of lymphadenopathy and severe fatigue. However, eight years later, his disease has not progressed, he is stable, and shows clinical signs of improvement. His latest T4 helper cell count was 382 (20%), somewhat below normal; however, the count has been stable for at least a year and a half. The latest T4 lymphocyte count for patient #15 was 576 (24%), slightly below normal, but consistent with the patient's good health.

The findings support the conclusion that patient #15 currently carries a nonpathogenic variant of HIV, that he has transmitted the variant virus to patient #16 and that the superinfection of patient #16 with nonpathogenic variant HIV has prevented the further deterioration of his immune system and has resulted in his currently improving condition. To the extent that any pathogenic virus of patient #16 has been transmitted to patient #15, the latter is not susceptible because he is already infected with the more competitive, nonpathogenic variant.

The patient identified in the present application as #16 was identified in U.S. Patent Application 07/540,529, filed June 16, 1990, as Patient #24. The identification number was changed in the present case to make it consistent with patient records in the clinical study.

Example 4. Clinical Improvement after Superinfection with Nonpathogenic HIV Variant.

Eleven severely immuno-compromised patients were each inoculated with a single injection of whole blood from an HIV-positive donor who has been asymptomatic over a long period of time (at least 11 years). Patients were monitored weekly for 12-16 weeks after inoculation. All HIV anti-viral drugs were discontinued prior to and during the study except for one

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5 patient who received brief treatment with interferon- α 2b for
Kaposi's sarcoma. No adverse affects were observed after
inoculation although one patient regressed and died 98 days
later. Five patients improved clinically, one remained stable
and the remaining four experienced a mixed response. In
addition to monitoring the apparent state of health in the
patients, estimates of capacity for cell-mediated immunity and
the level of humoral response to HIV was also monitored, thus
providing objective measurement of effect of the inoculation
10 with the putative nonpathogenic HIV variant.

15 The donor of the whole blood containing the putative
nonpathogenic HIV variant was Patient #1 as described in
Example 1. The donor was further tested to insure that neither
syphilis nor hepatitis or any other active infection would be
transmitted to members of the test population.

20 Members of the test population had already declined to
a state of severe immunosuppression and included individuals
who either could not tolerate AZT or DDI or in whom these drugs
had been ineffective, and had shown progressive T4 cell loss
despite standard therapies (mean T4 cell count 66, range 5-
132). Each had marked depletion of circulating anti-HIV
antibodies as well as severely impaired cell-mediated immunity
(as measured by delayed-type hypersensitivity skin test
reaction to eight test antigens). The test population ranged
25 in age from 26-44, and was composed of ten men and one woman.
All patients provided informed written consent prior to
entering the study.

30 The immunological state of each test patient was
determined prior to inoculation with donor blood to determine
baseline measurements. Each test patient was then monitored
weekly for 12-16 weeks after inoculation. Each evaluation
included clinical history, physical examination and a
laboratory profile including complete blood count (CBC),

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platelet counts, sedimentation rate, biochemistry and electrolyte profiles, Beta-2 microglobulin, p24 antigen, lymphocyte subset panels (including NK cell and CD8 subsets). Each test patient was also skin-tested (to measure DTH) at the onset of the study against eight antigens (Merieux CMI plus mumps) and this was repeated twelve to sixteen weeks after inoculation. Circulating antibody production against nine HIV-1 antigens was measured at baseline and at weekly intervals after inoculation. Tables 2-6 display the results of this testing.

Unrelated to clinical outcome, all laboratory values including T4 cell counts, remained stable, except for an increase in Beta-2-microglobulin in all eleven test patients. This suggests that a rebound in immune system function can be seen before actual cell numbers increase.

Within the first seven weeks after inoculation, a marked increase in total HIV non-core antibody production (against gp160, gp120, p64, p53 and p31) was noted in 10 of 11 patients. Levels of antibodies specific for combined core proteins (p55, p24 and p15) also increased markedly in all eleven test patients. Five of the 10 patients developed high levels of antibodies against p15. Four of those five patients remained strongly positive for antibodies directed against p15, and this aspect of the response to inoculation correlated positively with marked clinical improvement.

Six of the eleven test patients remained clinically stable or improved, developing no opportunistic disease (either as recurrence or as new onset), experiencing improved appetite, weight gain, decreased fatigue and increased stamina, allowing a return to normal exercise and daily routine. Each claimed an increased sense of well-being. At about eleven months after the initial inoculation with blood containing the nonpathogenic variant HIV, nine of the test patients remain in the study.

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Four patients have improved in their conditions as compared with their status at the start of the study.

For example, IMM #24 had experienced severe migrating arthralgias of two years' duration. These symptoms disappeared within twelve weeks after inoculation, allowing him to discontinue his previous high dosages of non-steroidal anti-inflammatory agents.

Patient #37 entered the study with a single Kaposi's sarcoma (KS) lesion of the skin. During the post-inoculation monitoring this lesion was observed to wax and wane in size and shade, but remained essentially stable.

Four of the eleven patients displayed mixed responses, improving with regard to some clinical problems, in well-being and in stamina, but continuing to experience major disease.

Patient #31 had been previously treated for KS (mucocutaneous) with Interferon-alpha-2b from 1987-1989 with complete remission for one year. KS lesions did not recur after interferon treatment was discontinued. For several years prior, he had experienced mild peripheral neuropathy and had suffered multiple episodes of severe acute pancreatitis associated with hypertriglyceridemia. During the sixteen weeks of post-inoculation monitoring, he experienced improved appetite, stamina and regression of the neuropathy. He was hospitalized twice, however, for brief and mild episodes of pancreatitis. He was able to resume normal activities after each episode including extensive travel. At nearly one year into the study, he is leading a normal life.

Patient #25 had developed KS of the cervical lymph nodes, tonsil, hard palate and skin one month prior to inoculation. He experienced increased stamina and with gain during the course of the study, but KS lesion size and number

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continued to increase and as a result, at six weeks after inoculation, he was treated with Interferon-alpha-2b (50 MU I.V. daily for 14 days) with moderate regression in lesion size for skin and hard palate and a complete regression of the tonsillar lesion. Interferon was discontinued after 14 days because of extreme fatigue. Four weeks later (at twelve weeks post-inoculation), a second course of interferon therapy was instituted (25 MU I.V. three times per week for 14 days), resulting in moderate regression of all lesions. The patient continued to experience weight gain and increased stamina, as well as return of nocturnal erections; he was able to engage in all normal work activities. He has since regressed.

Patient #30 experienced increased stamina and marked diminution in previously debilitating arthralgias and myalgias, but developed symptoms consistent with Sjögren's Syndrome, a single small KS lesion of the gingival mucosa at fourteen weeks after inoculation and a small CMV lesion of the retina. He was treated only with high dose gamma globulin for two weeks, resulting in complete regression of his retinal lesion at follow-up.

Patient #32 had previously suffered multiple episodes of pneumothorax, Pneumocystis carinii pneumonia and cytomegalovirus (CMV) retinitis with the loss of vision in one eye. During the course of the study, he experienced an episode of septicemia, associated with an infected central venous catheter, with a brief episode of weight loss, impaired stamina and depression. He recovered from these problems, but the CMV retinopathy continued to progress despite gancyclovir therapy. Surprisingly, however, almost all CMV activity (90%) resolved 16 weeks after inoculation. During the period after the second inoculation, this patient experienced a regression in AIDS symptomology, and ultimately ended his life.

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5 Patient #23, a pre-terminal female patient, aged 29, regressed and died. She had suffered an episode of critical pancreatitis prior to the entry into the study group, following which she developed CMV retinopathy, and magnetic resonance
10 imaging (MRI) results and central spinal fluid (CSF) protein consistent with HIV or CMV. She experienced an unusual and dramatic improvement in mental status three days after inoculation, which lasted for six weeks. She was subsequently hospitalized with severe anemia, erosive reflex esophagitis, bleeding ulcers and progressive encephalopathy. She died 14 weeks after inoculation.

15 Table 2 summarizes the clinical outcome results for the test population, along with the changes in non-core and core antibody levels. Increase in p15-specific antibody correlates with clinical improvement. A lack of a p55-specific antibody response correlates with regression or mixed clinical response. A marked increase in p24-specific antibody occurred in the two patients, one with minimal clinical improvement and one with stabilized condition, during the post-inoculation study period.
20 Tables 2 and 3 chronicle the antibody responses to particular HIV proteins before inoculation and during a 16 week post inoculation period. Table 4 illustrates the rebound of the cellular immune response, as measured by delayed-type hypersensitivity skin testing sixteen weeks after inoculation. Table 5 compares natural killer, cytotoxic killer, CD8 and CD8
25 suppressor cells in the AIDS patients inoculated with the nonpathogenic HIV with normal ranges. Over the 16 week monitoring period following inoculation, there was no significant effect on cell numbers, as reported in Table 5.

30 It should be noted that finding a patient who is asymptomatic for AIDS and whose blood plates out productive HIV generating a strong (3+) antibody response for all HIV protein epitopes and especially core protein epitopes, does not per se prove that the strain is nonpathogenic. Criteria for a

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nonpathogenic viral strain include 1) the viral strain does not kill T4 lymphocytes in vitro, 2) the viral strain propagates in culture relatively slowly, 3) it elicits an antibody response to HIV, particularly with antibodies specific for viral core proteins and 4) after inoculation into an immunocompromised AIDS patient, antibody levels increase, clinical well-being returns and cell-mediated immunity returns. These are all indications that the variant strain is either less virulent than the wild-type virus or is avirulent. A further criterion, but not an absolute criterion, for choosing a potential donor is the ability of serum to bind to 15 and/or 17kDa doublet proteins on Western blots of HIV proteins. The clinical improvement in patients inoculated with such a nonpathogenic viral strain shows that it is also competitive with resident pathogenic viral strains.

The findings described above describing neurological improvement, arthritic relief and partial restoration of immune function may be explained by viral infection of more than one cell type: i.e., macrophage, T cells, B cells and cells involved in the complement pathway or there may be indirect effects of HIV or of superinfecting nonpathogenic HIV variants. The return of B cell-mediated immunity, particularly with the production of antibodies specific to core epitopes (p55, p24 and p15), suggests that infection and colonization by the inoculated virus occurred. This is supported by the fact that few of the treated patients had antibodies to p15 prior to inoculation and almost all generated some anti-p15 antibody during the evaluation period following inoculation. In one patient (#26) p15 antibody increased six weeks after inoculation just as p24 antigen was decreasing, suggesting that one or more p15 peptide epitopes were neutralizing. This also suggests that the p15 peptide epitope(s) (for example, as a synthetic peptide coupled to a carrier protein or as a recombinant protein) could be an immunogen for inducing neutralizing antibodies to HIV, and thus, antibodies protective

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5 against AIDS. In fact, Tables 4 and 7-8 show that the
parameter most closely associated with improved clinical
condition was the positive response in the skin test. Another
parameter which was positively, but not absolutely, correlated
was increased levels of antibody specific for p15 and/or p17
doublet. Furthermore, in principle, a nonpathogenic variant
HIV virus described herein could be used as a live virus
vaccine since all eleven test patients showed increased
antibody production, at least initially, to both viral core and
10 envelope proteins.

15 The correlation of p15-specific and/or p17-specific
antibody with clinical well-being suggest strongly that the
viral strains which induces the production of anti-15 and/or
anti-17 antibody to HIV are not clinically harmful and,
therefore, can be considered nonpathogenic in a clinical sense.
It is nonpathogenic in a cellular sense (in vitro) in that it
does not kill T4 cells in culture.

20 Applicants believe that this is the first instance
where the return of the delayed-type hypersensitivity skin test
reaction in AIDS patients was observed; in this case it
occurred after inoculation with blood containing a
nonpathogenic HIV variant. This is a strong indicator that
cell-mediated immunity is returning and that the cells
responsible for this immunity are both regenerating and/or are
25 protected from destruction by wild-type HIV.

30 The pattern of stability of T4 cell numbers over twelve
to sixteen weeks in the test population suggests that the
expected increase in the number of T4 cells will take much
longer than three months, and that T4 number and T4/T8 ratio
do not correlate with changes in clinical status during a brief
period of evaluation. In fact, Patient #23 (who died) had the
largest T4 increases (from 120 at baseline to 208 in week 3).
The decrease in T8 cells, in the absence of concomitant

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reduction in number of percentage of T4 cells, is considered a good sign in that it more closely resembles a normal T4/T8 ratio. This may be correlated with the reduced arthralgias seen in this study.

5 About three months after the initial injection of donor
blood containing the nonpathogenic variant HIV, the ten
surviving test group members received second injections of the
10 IMM-1 donor blood. At about five and eleven months into the
study, the delayed-type hypersensitivity (DTH) skin test
reactions were again examined. Tables 7 and 8 present test
results taken at about five and eleven months into the study,
respectively. In all patients, the DTH response was increased
over that at the initiation of the study.

15 Entering the twelfth month into the study, four
patients are improved in their general health: #33, #27, #40
and #31. The four remaining patients exhibited a mixed
response or had regressed. At 13-14 weeks into the study,
Patient #23 died of CMV-related bleeding esophagitis. Patient
#32 regressed, and committed suicide 44 weeks into the study.
20 Patient #25 left the study after about six months when he
relocated to another city. Patient #25 has regressed after
earlier improvement, and was returning to antiviral therapy.

25 The reported general clinical well-being comes not only
from the clinician's evaluation but also from positive comments
by the test patients themselves. While this may reflect a
placebo affect, objective measures such as decreased analgesic
dosages, weight gain, increased exercise tolerance, improved

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stamina and restoration of humoral and mediated cell immunity as measured by DTH support the clinical impression of improved health.

5 When one considers the beneficial effects of Karpas' passive immunization treatments of AIDS patients, one might consider the possibility that the positive results noted herein could be explained by transferring neutralizing antibodies. Karpas et al. (1988) Proc. Natl. Acad. Sci. USA 85:9234-9237 showed that at least 500 ml of antibody-containing serum was
10 needed for positive results, and that those positive results do not generally last more than one month. Monthly transfusions would thus usually be needed to continue the positive "Karpas effect."

15 It is proposed herein that serial screening for clinical and cellular nonpathogenicity as well as selection for competitiveness against pathogenic strains will result in the identification of a more highly competitive phenotype. The patient who becomes clinically "well" then carries the most competitive strain of a nonpathogenic variant. The recipient
20 then becomes the new donor. One can continue to monitor the evolution of more competitive strains in this manner and select for the least pathogenic and most competitive variant by observing and screening for the most rapid return of clinical well-being. The identification of a more competitive strain

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involves a selection process while the identification of a nonpathogenic strain involves a screening process.

5 The competitiveness of an HIV variant as compared with the pathogenic HIV strain can be assessed using, for example, mixed and individual infections of cells in vitro. If variant and pathogenic strains are equally competitive, then a mixed infection with variant and pathogen at a 1:1 ratio should result in a 50% reduction in cell killing. If, however, the variant is more competitive than pathogen, then fewer cells
10 should be productively infected by pathogen than with the variant, and there should be greater than a 50% reduction in cell killing. The converse should be true in cases where the pathogenic virus is more competitive than the nonpathogenic variant.

15 One explanation for the varied clinical results in this study may be that the healthiest patients responded best and the sickest patients responded the least. Molecular competition appears to have occurred in all patients by virtue of the renewed production of core p15-specific antibody. The
20 inability to maintain core p15-specific antibody production may reflect the degree of immunologic deterioration at end stage disease.

 In this study, approximately 50% of the test patients experienced a sustained restoration of antibody production

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after one inoculation with a nonpathogenic HIV variant. From a public health perspective, this result suggests a method for mimicking and accelerating the natural termination of an epidemic. Therefore, one can speculate that the introduction of this strain into the population can reverse the course of this epidemic, decreasing the financial and social burden, extending the effective latency period and giving the research community more time to develop completely nonpathogenic, non-reverting highly competitive strains.

The present invention is applicable to a wide range of viruses of either humans or animals. The strategies for seeking nonpathogenic variants must be modified to take account of each virus' mode of infection and clinical course. Such adaptations and modifications will be expedients which those skilled in the art will understand how and why to apply in each circumstance. The use of nonpathogenic variant virus as either live or heat-killed vaccines will also vary in detail, depending on the virus and the animal or human to be immunized. The techniques of superinfection can also be varied, based on knowledge of normal routes of infection for each type of virus, as those skilled in the art will readily appreciate. In the case of HIV, method of treatment by superinfection with a nonpathogenic variant strain provides a low risk, inexpensive means to control the course of the disease in those who are afflicted. Both subjective observations and objective clinical data indicate that inoculation with nonpathogenic HIV variants

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leads to an improvement in the state of a symptomatic AIDS patient's immune function.

INDUSTRIAL APPLICABILITY

5 Nonpathogenic variant viruses, such as those disclosed herein, will be useful in the prophylaxis and treatment of disease. Specifically exemplified nonpathogenic variant HIV strains, which are more competitive than the pathogenic strains of AIDS patients, will be useful in the treatment of AIDS.

Table 2. Clinical Outcome for Test Population Inoculated with Non-Patogenic HIV Vaccine

DOI	Clinical Outcome	Total Baseline Core Ab (Scale 0-9)	High Weekly Total Core Ab (Scale 0-9)	Core Ab Mean (Scale 0-9)	p15 Baseline • HWL (Scale 0-3)	p24 Baseline • HWL (Scale 0-3)	p55 Baseline • HWL (Scale 0-3)	Total Non-core (Scale 0-18) (gp160, gp120, p64, p53, gp41, p31)	Total Ab Baseline • HWL Scale (0-27)
IMM-33 (M/28)	1986 Marked Improvement	3.5	6.5	5.6	0.5 • 3 Marked increase held	2 • 3 Moderate increase held	1 • 2 Moderate increase not held	14 • 18	17.5 • 24
IMM-40 (M/34)	1979 Marked Improvement	2.0	8.0	4.3	0.5 • 3 Marked increase held	1 • 2 Moderate increase held	0.5 • 3 Marked increase not held	18 • 18	20 • 26
IMM-26 (M/29)	1984 Moderate Improvement	2.0	7.5	4.2	0 • 3 Marked increase held	1 • 1 No change	1 • 3 Marked increase held	13 • 18	15 • 25
IMM-27 (M/33)	1979 Moderate Improvement	3.0	6.5	4.3	0 • 3 Marked increase held	2 • 3 Moderate increase not held	1 • 2 Moderate increase not held	15 • 17	18 • 23.5
IMM-24 (M/26)	1987 Minimal Improvement	2.0	4.5	2.5	0.5 • 0.5 No change	0.5 • 2 Marked increase held	1 • 2 Moderate increase held	10 • 13	12 • 15.5
IMM-37 (M/43)	1985 Stable	0.5	3.0	1.7	0 • 0.5 Minimal increase held	0.5 • 2 Marked increase held	0 • 0.5 Minimal increase not held	11 • 15.5	11.5 • 17.5
IMM-31 (M/44)	1983 Mixed	0.0	2.5	1.4	0 • 2 Moderate increase not held	0 • 1 Minimal increase not held	0 • 0.5 No change	11 • 18	11 • 20
IMM-25 (M/28)	1983 Mixed	3.0	5.0	3.7	0 • 1 Minimal increase held	2 • 3 Moderate increase held	1 • 1 No change	11 • 15	14 • 19
IMM-30 (M/35)	1986 Mixed	1.0	1.5	1.1	0 • 0.5 Minimal increase not held	1 • 1 No change	0 • 0 No change	17 • 18	18 • 19.5
IMM-32 (M/39)	1979 Mixed	3.0	4.0	1.9	2 • 3 Moderate increase not held	1 • 1 No change	0 • 0.5 No change	10 • 13	13 • 17
IMM-23 (F/29)	1986 Regressed	1.0	2.5	1.1	1 • 2 Moderate increase not held	0 • 0.5 No change	0 • 0 No change	12.5 • 17	13.5 • 18

Legend: DOI= date of infection
 HWL= highest weekly level
 Ab= Antibody

Ab Changes: minimal = 5
 moderate = 1
 marked = 2+

held = maintained for 4+ weeks

[illegible]

		GP160	GP120	P64	P55	P53	GP41	P31	P24	P15	Total
IMM-26	6/5										
(7/10-1cc #1)	7/10	3	3	1	1	1	2	3	1	0	15
	7/18	3	3	1	1	2	3	2	0	0	15
	7/26	3	3	2	2	2	3	3	1	0	19
	8/1	3	3	2	2	2	3	3	0.5	0	18.5
	8/8	3	3	1	1	1	3	2	0	0	14
	8/15	3	3	2	2	2	3	3	0.5	0	18.5
	8/22	3	3	2	2	2	2	2	0.5	0	16.5
	8/29	3	3	2	2	2	3	3	1	3	21
	9/5	3	3	2	2	2	3	3	1	3	22
	9/12	3	3	3	3	3	3	3	1	3	25
	9/19	3	2	2	2	1	3	3	0.5	3	19.5
	9/26	3	3	2	2	1	3	2	0.5	3	19.5
	10/3	3	1	2	2	2	3	3	0.5	3	19.5
	10/24	3	3	2	2	1	3	3	1	3	21
										Mean:	19
IMM-27	5/29	3	3	2	2	2	3	2	2	0	19
(7/10-1cc #1)	6/28	3	3	2	1	1	3	3	2	0	18
	7/18	3	3	2	1	1	3	2	2	0	17
	7/26	3	3	3	2	2	3	3	2	0	21
	8/1	3	3	2	1	1	3	3	2	0	18
	8/8	2	2	2	1	1	2	3	2	0	15
	8/15	3	3	3	2	2	3	3	2	0	21
	8/22	3	3	2	1	2	3	3	2	0	19
	8/29	3	3	3	0	2	3	3	3	3	23
	9/5	3	3	3	0	1	3	2	2	3	20
	9/12	3	3	3	0	1	3	2	2	3	20
	9/19	3	3	3	0	1	3	3	2	3	21
	9/26	3	3	3	0.5	2	3	3	3	3	23.5
	10/3	3	3	3	0.5	1	3	2	1	2	18.5
	10/24	3	3	3	0.5	2	3	2	2	3	21.5
										Mean:	20
IMM-32	6/1										
(7/19-.5cc #1)	7/3	3	3	0.5	0	0.5	2	1	1	2	13
	7/26	2	2	0.5	0	0.5	2	1	1	2	11
	8/1	3	3	0.5	0	0.5	2	1	1	2	13
	8/8	3	3	1	0.5	1	3	2	0.5	3	17
	8/15	3	3	0.5	0	0.5	3	2	1	3	16
	8/22	3	3	0.5	0	0.5	3	2	1	2	15
	8/29	3	3	1	0	0.5	3	1	0.5	0.5	12.5
	9/5	3	3	0.5	0	0	3	0.5	0.5	0	10.5
	9/12	3	2	0.5	0	0	3	0.5	0.5	0.5	10
	9/19	3	3	0.5	0	0	2	0.5	0.5	0	9.5
	9/25	3	2	0.5	0	0	3	0.5	0.5	0.5	10
	10/3	3	2	0.5	0	0	3	0.5	0.5	0.5	10
	10/10	3	3	1	0	0.5	3	1	1	0.5	13
										Mean:	12

[illegible]

[illegible]

Table 4. Delayed-type Hypersensitivity Responses after Inoculation with HIV Variant

	Delayed Type Hypersensitivity Skin Test				Follow-up Test (12-16 wks after inoculation)		Day 1	Day 2
	Baseline Test	Day 1	Day 2					
IMM-33 (7/19-5cc IMM-1)	7/26 3+ (tetanus, proteus, tuberculin)	3/8.5mm	2/5mm	10/24 4+ (tetanus, diphtheria, proteus, mumps)	4/15.5mm	3/9mm		
IMM-40 (8/14-2cc IMM-1)	8/15 All Negative	0	0	10/24 2+ (tuberculin, mumps)	2/6mm	2/4.25mm		
IMM-26 (7/10-1cc IMM-1)	7/18 2+ (tetanus, proteus)	2/13mm	2/10mm	10/24 5+ (mumps, diphtheria, streptococcus, trichophyton, proteus)	5/27mm	4/12.5mm		
IMM-27 (7/10-1cc IMM-1)	7/19 1+ (proteus)	1/7mm	1/7mm	10/24 5+ (streptococcus, tuberculin, candidiasis, proteus, mumps)	5/21mm	5/21mm		
IMM-24 (7/10-1cc IMM-1)	7/26 All Negative	0	0	10/24 3+ (tuberculin, proteus, mumps)	3/16.5mm	2/4mm		
IMM-37 (7/24-1cc IMM-1)	7/25 All Negative	0	0	10/24 2+ (proteus, tetanus)	2/12mm	2/4mm		
IMM-25 (7/10-1cc IMM-1)	7/19 1+ (proteus)	1/5.5mm	1/5.5mm	10/24 3+ (tuberc, proteus, mumps)	3/14.5mm	3/9mm		
IMM-30 (7/19-5cc IMM-1)	7/26 All Negative	0	0	10/24 5+ (tetanus, candidiasis, trichophyton, proteus, mumps)	4/14.5mm	3/13mm		
IMM-32 (7/19-5cc IMM-1)	7/27 2+ (proteus, mumps)	2/19mm	2/7mm	10/31 4+ (tetanus, diphtheria, tuberculin, proteus)	4/12mm	4/9mm		

Dates of inoculation and volumes of whole blood inoculum are identified.

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Table 5. T-Cell Populations After Inoculation with Non-Pathogenic HIV Variant

Natural Killer: 3.2—22.2
Cytotoxic Killer: 1.0—7.8

Cytotoxic CD8 Fractionation: Killer: 10-31% (200-700)
Suppressor: 4-27% (40-630)

		Natural Killer	Cytotoxic Killer	CD8 Killer	CD8 Suppressor
IMM-25	9/5	9%	4%		
(7/10-1cc IMM-1)	9/12	20%	2%	200 (31%)	180 (28%)
	9/21				
	9/26	23%	6%		
	10/3	23.6%	4.8%		
IMM-23	9/12	17%	10%	530 (42%)	110 (9%)
	9/19	14%	5%	280 (32%)	150 (17%)
	9/26	22%	10%		
IMM-26	9/5	18%	2%		
(7/10-1cc IMM-1)	9/12	16%	3%	260 (41%)	110 (17%)
	9/19	24%	4%	450 (46%)	120 (12%)
	9/26	24%	2%		
	10/3	20%	2%	560 (55%)	100 (10%)
IMM-27	9/5	15%	2%		
(7/10-1cc IMM-1)	9/12	14%	4%	150 (19%)	300 (39%)
	9/19	13%	3%	130 (13%)	460 (47%)
	9/26	15%	4%		
	10/3	19%	3%	150 (18%)	320 (38%)
IMM-32	9/5	14%	1%		
7/19-.5cc IMM-1)	9/12	25%	2%	30 (9%)	130 (43%)
	9/19	31%	2%	40 (13%)	130 (41%)
	9/25	29%	1%		
	10/3	17%	1%	60 (14%)	240 (53%)
IMM-30	9/5	13%	5%		
7/19-.5cc IMM-1)	9/12	11%	6%	650 (48%)	360 (27%)
	9/19	14%	3%	400 (45%)	250 (28%)
	9/26	9%	1%		
	10/3	16%	6%	390 (42%)	280 (31%)
IMM-33	9/5	20%	4%		
7/19-.5cc IMM-1)	9/12	21%	4%	970 (57%)	150 (9%)
	9/19			940 (51%)	240 (13%)
	9/26	20%	2%		
	10/3	18%	4%	910 (54%)	200 (12%)

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Table 5. (Page 2)

		Natural Killer	Cytotoxic Killer	CD8 Killer	CD8 Suppressor
IMM-37	9/5	45%	1%		
(7/24-1cc IMM-1)	9/12	41%	1%	340 (27%)	90 (7%)
	9/19	44%	1%	410 (26%)	110 (7%)
	9/26	45%	1%		
	10/3	34%	2%	380 (31%)	70 (6%)
IMM-40	9/5	25%	4%		
(8/14-2cc IMM-1)	9/12	21%	3%	350 (39%)	80 (9%)
	9/19	21%	4%	330 (38%)	100 (11%)
	9/26	23%	1%		
	10/3	21%	3%	390 (45%)	50 (6%)
IMM-24	9/26	15%	6%		
(7/10-1cc IMM-1)	10/3	11%	8%	440 (58%)	70 (9%)
IMM-31	9/26	4%	1%		
7/19-.5cc IMM-1)					

	WBC	Total	Hg	Platelets	Sed Rate
		Lymph		(000s)	
7/10	3.2	900	16.9	189	5
7/18	2.5	1000	16.5	213	2
7/26	2.8	900	15.4	250	5
8/1	2.2	600	13.9	187	3
8/8	2.2	800	13.7	164	2
8/15	2.2	700	13.6	155	1
8/22	2.2	600	13.5	145	3
8/29	2.6	700	13.4	129	2
9/5	2.2	500	14.6	130	3
9/12	1.5	600	14.4	90	4
9/21	1.8	800	14.9	67	15
9/26	2.3	700	13.2	154	14
10/4	2.0	700	14.0	134	N/A
10/22	3.3	1200	14.2	114	8

7/10	3.6	1100	14.6	158	5
7/18	3.7	1200	14.8	172	4
7/26	2.6	900	14.2	186	5
8/1	3.6	1000	13.0	143	5
8/8	3.8	900	14.5	145	7
8/15	2.1	800	13.5	158	5
8/22	2.5	700	13.1	111	8
8/29	3.6	900	14.0	160	14
9/5	2.9	900	13.3	140	9
9/12	2.5	600	12.7	104	12
9/19	2.9	1000	13.1	162	6
9/26	3.6	1500	12.9	120	8
10/3	3.0	1000	12.7	120	9
10/24	3.0	1200	13.2	151	3

7/10	3.8	1200	14.7	261	2
7/18	3.9	1100	15.4	235	3
7/26	4.9	1200	15.7	239	2
8/1	6.0	1200	15.7	204	2
8/8	7.0	800	17.1	177	2
8/15	4.3	800	16.3	188	4
8/22	3.0	1100	15.4	181	3
8/29	3.8	900	15.6	178	4
9/5	4.3	1000	15.6	186	3
9/12	3.9	800	15.5	163	3
9/19	5.4	1000	14.6	212	4
9/26	4.6	1200	14.9	177	5
10/3	4.0	800	15.3	195	3
10/24	4.1	1000	17.0	212	5

7/10	5.3	600	15.3	159	2
7/18	5.5	1100	13.9	108	1
7/26	4.6	1400	13.4	171	28
8/1	4.0	1600	13.8	175	24
8/8	4.0	1600	13.8	175	24
8/15	5.7	3000	13.5	186	33
8/22	3.2	1200	13.3	156	1
8/29	3.8	2100	13.5	169	8
9/5	3.9	1400	14.4	157	7
9/12	2.5	1400	12.5	111	14
9/19	2.7	900	11.8	142	30
9/26	2.6	1100	10.9	127	65
10/3	2.7	900	11.0	107	127
10/24	2.6	800	12.2	171	36

7/10	5.6	1700	14.4	203	11
7/18	5.1	2100	14.0	258	9
7/26	4.7	1800	12.4	224	6
8/1	5.1	1500	12.9	218	12
8/8	4.8	1300	12.9	229	9
8/15	4.7	1600	13.5	207	9
8/22	4.4	1600	13.5	199	3
8/29	3.9	2400	13.1	199	9
9/5	5.4	2100	12.6	147	7
9/12	5.0	1700	12.6	204	3
9/19	5.1	1800	12.5	163	5
9/26	5.3	2000	12.9	176	6
10/3	5.1	1700	13.1	169	6
10/24	5.7	2000	14.5	228	5

7/10	3.4	700	13.1	285	13
7/18	3.5	900	13.6	225	9
8/1	3.0	800	14.0	164	5
8/22	3.0	500	14.9	190	4
8/29	4.1	500	14.9	190	4
9/5	4.3	1000	14.7	177	2
9/12	3.3	900	14.6	164	2
9/19	3.0	900	14.7	153	3
9/26	4.2	1100	14.6	138	1
10/3	2.3	900	13.7	103	3
10/10	3.3	1300	15.2	140	3
10/24	3.2	1000	13.6	154	4

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	3.2	900	16.9	189	5
7/18	2.5	1000	16.5	213	2
7/26	2.8	900	15.4	250	5
8/1	2.2	600	13.9	187	3
8/8	2.2	800	13.7	164	2
8/15	2.2	700	13.6	155	1
8/22	2.2	600	13.5	145	3
8/29	2.6	700	13.4	129	2
9/5	2.2	500	14.6	130	3
9/12	1.5	600	14.4	90	4
9/21	1.8	800	14.9	67	15
9/26	2.3	700	13.2	154	14
10/4	2.0	700	14.0	134	N/A
10/22	3.3	1200	14.2	114	8

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	3.6	1100	14.6	158	5
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8/8	3.8	900	14.5	145	7
8/15	2.1	800	13.5	158	5
8/22	2.5	700	13.1	111	8
8/29	3.6	900	14.0	160	14
9/5	2.9	900	13.3	140	9
9/12	2.5	600	12.7	104	12
9/19	2.9	1000	13.1	162	6
9/26	3.6	1500	12.9	120	8
10/3	3.0	1000	12.7	120	9
10/24	3.0	1200	13.2	151	3

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	3.8	1200	14.7	261	2
7/18	3.9	1100	15.4	235	3
7/26	4.9	1200	15.7	239	2
8/1	6.0	1200	15.7	204	2
8/8	7.0	800	17.1	177	2
8/15	4.3	800	16.3	188	4
8/22	3.0	1100	15.4	181	3
8/29	3.8	900	15.6	178	4
9/5	4.3	1000	15.6	186	3
9/12	3.9	800	15.5	163	3
9/19	5.4	1000	14.6	212	4
9/26	4.6	1200	14.9	177	5
10/3	4.0	800	15.3	195	3
10/24	4.1	1000	17.0	212	5

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	5.3	600	15.3	159	2
7/18	5.5	1100	13.9	108	1
7/26	4.6	1400	13.4	171	28
8/1	4.0	1600	13.8	175	24
8/8	4.0	1600	13.8	175	24
8/15	5.7	3000	13.5	186	33
8/22	3.2	1200	13.3	156	1
8/29	3.8	2100	13.5	169	8
9/5	3.9	1400	14.4	157	7
9/12	2.5	1400	12.5	111	14
9/19	2.7	900	11.8	142	30
9/26	2.6	1100	10.9	127	65
10/3	2.7	900	11.0	107	127
10/24	2.6	800	12.2	171	36

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	5.6	1700	14.4	203	11
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8/8	4.8	1300	12.9	229	9
8/15	4.7	1600	13.5	207	9
8/22	4.4	1600	13.5	199	3
8/29	3.9	2400	13.1	199	9
9/5	5.4	2100	12.6	147	7
9/12	5.0	1700	12.6	204	3
9/19	5.1	1800	12.5	163	5
9/26	5.3	2000	12.9	176	6
10/3	5.1	1700	13.1	169	6
10/24	5.7	2000	14.5	228	5

WBC	Total	Hg	Platelets	Sed Rate	
	Lymph		(000s)		
7/10	3.4	700	13.1	285	13
7/18	3.5	900	13.6	225	9
8/1	3.0	800	14.0	164	5
8/22	3.0	500	14.9	190	4
8/29	4.1	500	14.9	190	4
9/5	4.3	1000	14.7	177	2
9/12	3.3	900	14.6	164	2
9/19	3.0	900	14.7	153	3
9/26	4.2	1100	14.6	138	1
10/3	2.3	900	13.7	103	3
10/10	3.3	1300	15.2	140	3
10/24	3.2	1000	13.6	154	4

Table 6. (Page 2)

T#	T#	Base 2	P24 Ant.	T#	T#	Base 2	P24 Ant.	T#	T#	Base 2	P24 Ant.	T#	T#	Base 2	P24 Ant.								
NAV-25 (7/10-loc #1)	7/10	18	504	2.1	56	NAV-26 (7/10-loc #1)	7/10	132	583	1.9	152	NAV-31 (7/19-5cc #1)	7/9	90	1404	N/A	27	NAV-37 (7/24-1cc #1)	7/6	119	704	1.5	0
Baseline	7/26	9	486	2.3	7	7/26	72	549	1.8	320	8/1	28	1092	N/A	40	8/7	91	585	3.1	0	0	0	0
	8/1	6	318	3.8	0	8/1	90	640	2.9	232	8/15	54	1422	4.2	54	8/22	60	492	3.3	9	9	9	9
	8/8	16	464	3.7	6	8/8	90	558	2.9	344	8/22	48	568	3.8	78	8/29	48	492	2.8	9	10	10	10
	8/15	14	378	3.9	10	8/15	96	432	3.3	156	9/6	60	720	3.9	84	9/5	70	546	3.1	11	11	11	11
	8/22	12	324	4.2	35	8/22	77	406	3.4	156	9/12	110	1628	4.1	78	9/12	65	481	3.2				
	8/29	14	357	3.6	40	8/29	63	504	2.7	62	9/20	36	936	N/A	104	9/19	80	592	3.3				
	9/5	10	250	5.0	60	9/5	99	513	2.7	66	9/26	64	1184	4.4	104	9/26	80	600	3.4				
	9/12	6	312	5.2	74	9/12	60	366	3.2	100	10/11	48	864	3.9	65	10/3	34	731	3.1				
	9/21	10	340	N/A	83	9/21	50	600	3.0	57													
	9/26	21	385	4.7	64	9/26	90	990	2.8	46													
	10/3	42	378	4.8	70	10/3	90	630	3.2	53													
	10/24	24	696	5.8		10/24	110	600	3.2														
NAV-24 (7/10-loc #1)	7/10	70	710	N/A	32	NAV-27 (7/10-loc #1)	7/18	678	804	1.8	0	NAV-30 (7/19-5cc #1)	7/26	77	759	1.5	34	NAV-40 (8/14-2cc #1)	7/26	35	378	2.1	32
	7/26	66	390	1.4	36	7/26	44	715	N/A	0	8/1	84	1022	4.6	10	8/15	32	456	4.1	5	5	5	5
	8/1	96	520	6.4	33	8/1	40	550	2.4	0	8/8	80	1184	3.7	0	8/22	45	432	3.6	5	5	5	5
	8/8	54	378	7.1	42	8/8	32	456	3.1	0	8/22	72	912	4.9	6	8/29	45	432	3.4	39	39	39	39
	8/15	60	390	6.6	44	8/15	32	456	3.1	0	9/5	84	1022	4.3	9	9/5	80	450	3.4	39	39	39	39
	8/22	60	372	6.9	61	8/22	33	671	3.2	0	9/12	80	970	3.5	11	9/12	27	450	3.6	124	124	124	124
	8/29	66	348	5.4	27	8/29	18	522	3.3	0	9/19	54	630	3.9	19	9/19	27	441	3.6	180	180	180	180
	9/5	88	682	5.8	40	9/5	40	590	3.6	0	9/26	55	781	3.8	28	9/26	33	561	4.0	132	132	132	132
	9/26	88	682	5.8	40	9/26	18	522	3.3	0	10/3	36	612	4.1	15	10/3	45	450	4.1	144	144	144	144
	10/3	64	552	6.7	208	10/3	32	464	3.8	0	10/24	40	536	4.5		10/24	30	440	4.0	4.3	4.3	4.3	4.3
	10/24	63	567	7.5		10/24	20	430	3.6	0													
NAV-23 (7/10-loc #1)	6/12	120	820	1.3	N/A	NAV-32 (7/19-5cc #1)	7/3	5	300	2.3	N/A	NAV-33 (7/19-5cc #1)	7/5	68	1088	1.8	0						
	7/18	110	561	N/A	23	7/26	5	340	1.9	6	8/1	105	1365	1.5	0								
	7/26	104	376	1.4	20	8/1	21	448	N/A	6	8/8	72	1188	2.3	0								
	8/1	208	546	3.8	45	8/8	12	384	5.0	8	8/15	90	990	2.6	0								
	8/8	138	874	4.0	34	8/15	8	244	3.9	10	8/22	80	806	2.6	0								
	8/15	42	282	5.3	164	8/22	24	196	7.3	6	8/29	120	1560	2.7	0								
	8/22	90	630	2.2	19	8/29	3	177	4.1	9	9/5	105	1373	2.6	0								
	8/29	128	576	2.3	0	9/5	20	145	3.6	0	9/12	51	1105	2.5	0								
	9/5			7.9	0	9/12	6	153	7.1	5	9/19	70	1120	2.3	0								
	9/12	91	585			9/19	3	168	4.4	8	10/3	110	1210	2.7	0								
	9/19	110	610			10/3	10	170			10/24	80	1260	2.7	0								
	9/26	126	387	5.0	24	10/24	4	240	4.6	9													
		110	420																				
		102	833	3.8	9																		

Table 7. Delayed Type Hypersensitive Skin Test Responses

			Follow-up Test #2 (about 5 months after inoculation #1)	Day 2
<hr/>				
IMM-33 (7/19-.5cc IMM-1) (10/24-2cc IMM-1)	12/12/90	8+ (tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, proteus, mumps)	8/41.5mm	
<hr/>				
IMM-27 (7/10-1cc IMM-1) (10/24-2cc IMM-1)	12/12/90	7+ (tetanus, streptococcus, candida, trichophyton, proteus, tuberculin, mumps)	7/29.5mm	
<hr/>				
IMM-40 (8/14-2cc IMM-1) (10/24-2cc IMM-1)	12/12/90	2+ (proteus, mumps)	2/19.3mm	
<hr/>				
IMM-31 (7/19-.5cc IMM-1) (11/14- IMM-1)	12/12/90	3+ (tetanus, streptococcus, proteus)	3/9.7mm	
<hr/>				
IMM-24 (7/10-1cc IMM-1) (10/24-2cc IMM-1)	12/12/90	4+ (tetanus, tuberculin, candida, proteus)	4/13.2mm	
<hr/>				
IMM-37 (7/24-1cc IMM-1) (10/24-2cc IMM-1)	12/12/90	4+ (tetanus, tuberculin, streptococcus, proteus)	4/17mm	

TABLE 7 (CONTINUED) (Follow-up Test #2)

IMM-26 (7/10-1cc IMM-1) (10/24=2cc IMM-1)	12/12/90	4+ (tetanus, proteus, tuberculin, mumps)	4/17.75mm
IMM-30 (7/19-.5cc IMM-1) (10/24-3cc IMM-01)	12/12/90	3+ (tetanus, proteus, diphtheria)	3/11.5mm
IMM-25 (7/10-1cc IMM-1) (10/24-2cc IMM-1)	12/12/90	3+ (tetanus, proteus, mumps)	3/16mm
IMM-32+ (7/19-.5cc IMM-1) (10/24-3cc IMM-1)	12/12/90	3+ (tetanus, proteus, mumps)	3/24mm

Table 8. Delayed Type Hypersensitive Skin Test Responses

		Follow-up Test #3 (about 11 months after inoculation #1)		Day 1	Day 2
IMM-33	5/23/91	8+ (tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, proteus, mumps)		8/43.7mm	
IMM-27	5/24/91	8+ (tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, proteus, mumps)		8/40.5mm	
IMM-40	5/24/91	6+ (tetanus, diphtheria, streptococcus, tuberculin, proteus, mumps)		6/23.5mm	4/8.5mm
IMM-31	5/24/91	6+ (tetanus, diphtheria, streptococcus, tuberculin, proteus, mumps)		6/37mm	6/20mm
IMM-24	5/24/91	3+ (tetanus, tuberculin, proteus)		3/7mm	
IMM-37	5/24/91	5+ (tetanus, diphtheria, streptococcus, proteus, mumps)		5/27mm	

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TABLE 8 (CONTINUED) (Follow-up Test #3)

IMM-26	5/24/91	4+ (tetanus, streptococcus, proteus, mumps)	4/17mm	4/8mm
IMM-30	5/24/91	3+ (tuberculin, proteus, mumps)	3/11mm	3/5.5mm

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CLAIMS

1. A method for isolating a nonpathogenic variant virus strain of a pathogenic virus comprising screening a population of individuals infected or at risk of being infected by the virus to identify those who are virus-positive and symptom-free for a period longer than the normal time for appearance of symptoms of disease caused by the virus, culturing the virus from said symptom-free, virus-positive individuals, then screening the cultured virus for characteristics of nonpathogenic variant virus and cloning any virus having characteristics of nonpathogenic variant virus.
2. A method for isolating a nonpathogenic variant HIV strain comprising screening an HIV-positive population of individuals to identify those who have been HIV-positive and symptom-free for at least 8 years, culturing HIV from the blood cells of said symptom-free HIV-positive individuals, testing the growth rate of individual HIV cultures and cloning slow-growing virus from said cultures whereby the nonpathogenic variant HIV strain is isolated.
3. A nonpathogenic variant HIV strain isolated by the method of claim 2.

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4. The nonpathogenic variant HIV strain of claim 3, wherein said strain is cultured from the blood cells of IMM-1.
5. The nonpathogenic variant HIV strain of claim 3, wherein said strain is cultured from the blood cells of one of IMM-29 and IMM-41.
6. A live HIV virus vaccine comprising a nonpathogenic variant HIV strain isolated by the method of claim 2 and a physiologically acceptable diluent.
7. A method of immunizing a human against AIDS comprising administering to said human an immunizing amount of a nonpathogenic variant HIV strain isolated by the method of claim 2.
8. An inactivated HIV virus vaccine comprising an inactivated, nonpathogenic HIV strain isolated by the method of claim 2 and a physiologically acceptable diluent.
9. A method of treating an HIV-infected patient to stabilize and/or improve the patient's clinical condition comprising superinfecting the patient with an infecting amount of a competitive nonpathogenic variant HIV strain.

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10. A method of treating an HIV-infected patient according to claim 9, wherein the competitive nonpathogenic HIV strain is cultured from blood cells of an individual who has been both HIV-positive and symptom-free for at least 8 years.
11. The method of treating an HIV-infected patient according to claim 10, wherein said individual is IMM-1.
12. The method of treating an HIV-infected patient according to claim 10, wherein said individual is one of IMM-29 and IMM-41.
13. A method of treating an HIV-infected patient to elicit an immune response against HIV core proteins comprising superinfecting the patient with an infecting amount of a competitive nonpathogenic variant HIV strain.
14. The method according to claim 13 wherein said variant HIV strain is cultured from blood cells of IMM-1.
15. The method according to claim 13 wherein said variant HIV strain is cultured from blood cells from one of IMM-29 and IMM-41.

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16. A method of treating an HIV-infected patient to increase cell-mediated immunity comprising superinfecting the patient with an infecting amount of a competitive nonpathogenic variant HIV strain.

5 17. The method of claim 16 wherein said variant HIV strain is cultured from blood cells of IMM-1.

18. The method of claim 16 wherein said variant HIV strain is cultured from blood cells from one of IMM-29 and IMM-41.

10 19. A method of making a highly adaptive nonpathogenic virus strain of a nonpathogenic variant virus comprising the steps of:

15 a) identifying a first locus in the nucleotide sequence of the nonpathogenic variant whereat a base change correlates with a change in phenotype from pathogenicity to nonpathogenicity;

20 b) identifying a corresponding second locus in the nucleotide sequence of the nonpathogenic variant where base-pairing between the first locus and second locus participates in forming secondary structure of DNA or RNA of the virus;

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c) making a second site mutation at the second locus such that the resulting interaction between the first and second locus preserves the secondary structure whereby a reversion to a pathogenic sequence at the first locus fails to base pair at the second locus, and;

d) transforming a host cell with DNA or RNA of the virus knowing the second site mutation, thereby producing a highly adaptive nonpathogenic virus.

10 20. A highly adaptive nonpathogenic HIV made by the method of claim 19.

15 21. A method of treating an HIV-infected patient to stabilize the patient's clinical condition comprising superinfecting the patient with an infecting amount of a highly adaptive nonpathogenic HIV made by the method of claim 19.

20 22. A method of immunizing a human against AIDS comprising administering to said human an immunizing amount of a highly adaptive nonpathogenic HIV strain made by the method of claim 19.

23. A method of treating cytomegalovirus infections and lesions in an HIV-infected patient comprising

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superinfecting the patient with an infecting amount of a competitive nonpathogenic variant HIV strain.

24. The method of treating cytomegalovirus infections and lesions in an HIV-infected patient according to claim 23, wherein said variant HIV strain is cultured from blood cells of IMM-1.

25. The method of treating cytomegalovirus infections and lesions in an HIV-infected patient according to claim 23, wherein said variant HIV strain is cultured from blood cells of one of IMM-29 and IMM-41.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US91/04374

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 07/01; A61K 35/76

U.S. Cl.: 435/239; 424/89, 93

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/239; 424/89, 93

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched ⁸

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1975+,
NTIS, AIDSLINE, CA SEARCH, BIOTECHNOLOGY ABSTRACTS 1982+

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P, Y/	EP, A, 0,381,146 (KOBAYASHI ET AL) 08 AUGUST 1990. See entire document.	2-4,6-7,9-11, 13-14,16-17, 23-24
P, Y/	The Journal of Experimental Medicine, Vol. 172, issued September 1990, S. H. Pincus et al, "Variants Selected by Treatment of Human Immunodeficiency Virus-Infected Cells with an Immunotoxin," pages 745-757. See entire article.	2-4,6-7,9-11, 13-14,16-17, 23-24
Y	Nature, Vol. 327, issued 11 June 1987, J. Salk, "Prospects for the control of AIDS by immunizing seropositive individuals," pages 473-476. See entire article.	2-4,6-7,9-11, 13-14,16-17, 23-24
Y	Annals of Internal Medicine, Vol. 110 No. 5, issued 01 March 1989, A. S. Fauci et al, "Development and Evaluation of a Vaccine for Human Immunodeficiency Virus (HIV) Infection," pages 373-385. See entire article.	2-4,6-7,9-11, 13-14,16-17, 23-24

^{*} Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not
considered to be of particular relevance

"E" earlier document but published on or after the international
filing date

"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or
other means

"P" document published prior to the international filing date but
later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

"X" document of particular relevance: the claimed invention
cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance: the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

17 AUGUST 1991

Date of Mailing of this International Search Report

03 OCT 1991

International Searching Authority

ISA/US

Signature of Authorizing Officer

JOHNNY F. RAILY II

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EP, A, 0,233,764 (FISHER ET AL) 26 AUGUST 1987. See entire document.	2-4, 6-7, 9-11, 13-14, 16-17, 23-24
Y	WO, A, 87/06259 (FISHER ET AL) 22 OCTOBER 1987. See entire document.	2-4, 6-7, 9-11, 13-14, 16-17, 23-24
Y	EP, A, 0,335,635 (MCCUNE ET AL) 10 APRIL 1989. See entire document.	2-4, 6-7, 9-11, 13-14, 16-17, 23-24
Y	EP, A, 0,315,459 (KATO ET AL) 10 MAY 1989. See entire document.	2-4, 6-7, 9-11, 13-14, 16-17, 23-24

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☒ Claim numbers 1, 19, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

Claims 1 and 19 are so vague and indefinite as to prevent a meaningful and thorough search.

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

2-4, 6-7, 9-11, 13-14, 16-17 and 23-24

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.